JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Application of Box-Behnken Experimental Design to Optimize the Extraction of Insecticidal Cry1Ac from Soil

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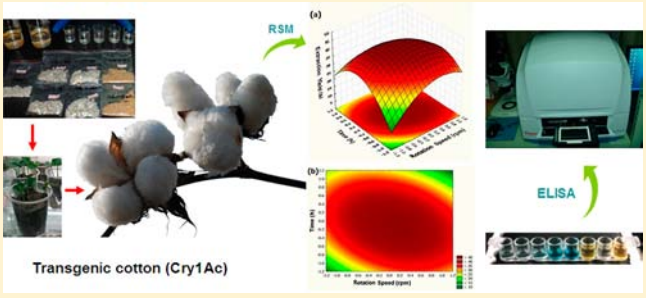
ABSTRACT: A validated method for analyzing Cry proteins is a premise to study the fate and ecological effects of contaminants associated with genetically engineered *Bacillus thuringiensis* crops. The current study has optimized the extraction method to analyze Cry1Ac protein in soil using a response surface methodology with a three-level-three-factor Box-Behnken experimental design (BBD). The optimum extraction conditions were at 21 °C and 630 rpm for 2 h. Regression analysis showed a good fit of the experimental data to the second-order polynomial model with a coefficient of determination of 0.96. The method was sensitive and precise with a method detection limit of 0.8 ng/g dry weight and relative standard deviations at 7.3%. Finally, the established method was applied for analyzing Cry1Ac protein residues in field-collected soil samples. Trace amounts of Cry1Ac protein were detected in the soils where transgenic crops have been planted for 8 and 12 years.



KEYWORDS: *response surface methodology, Box-Behnken design, Cry1Ac protein, transgenic Bt cotton, soil*

INTRODUCTION

Genetically engineered crops that express Cry proteins from *Bacillus thuringiensis* (Bt) are effective against lepidopteran pests such as *Helicoverpa armigera*.[1] Planting Bt crops demonstrated the advantages of the reduced needs of the broad spectrum chemical pesticides and the suitability for insect resistance management. Consequently, the cultivation areas of Bt crops experienced a rapid increase worldwide from 1.7 million hectares in 1996 when Bt crops were first commercialized to more than 66 million hectares in 2011.[2] In China, Bt cottons were planted on a large scale and almost completely replaced non-Bt cotton, which made China to be the second largest Bt cotton-growing country in the world.[1,3]

The widespread planting of Bt crops has raised concerns on their environmental risks.[4,5] Insecticidal Cry proteins produced by Bt crops may be released to soil through roots exudates, tasseling, or plant residues. As a consequence, persistence of Cry proteins in soil become an urgent concern.[6−11] After entering the soil, Cry proteins quickly sorbed to clay minerals[12] and humic substances,[13] which significantly reduced their availability to be degraded by the microorganisms.[14] Saxena et al.[7] reported that Cry proteins released from the root exudates of Bt corns accumulated in soil, and their insecticidal activity remained effective for over 180 d. Conversely, other researchers claimed that no Cry proteins were detectable in soils where Bt cottons had been planted for multiple years.[15] In addition to the arguments on the fate of Bt proteins, debates are going on for their ecological risks.[5,16] Although no conclusive decision has been drawn until now, previous studies showed that the residual Bt proteins might adversely affect soil biodiversity due to their toxicity to the invertebrates and microorganisms.[17] Therefore, more studies are needed to understand the fate and risks of soil-associated Bt proteins, and an effective and robust method for analyzing trace amounts of Cry proteins in soil is the premise of these types of research.

A variety of methods have been developed to assess Bt proteins, such as bioassays,[15] enzyme-linked immunosorbent assay (ELISA),[15,18] circular dichroism spectroscopy, and size-exclusion high performance liquid chromatography.[19] Within these methods, ELISA was the most commonly used detection method for Bt proteins due to its simplicity in operation, short quantification time, high sensitivity, and capacity for high throughput testing.[12,15] Before ELISA quantification, Cry proteins were required to be extracted from the soils,[15,18,20,21] and extraction efficiency differed markedly among soils with different characteristics, i.e., their mineralogy and clay contents.[22,23]

The objective of the current study was to develop and validate an extraction method for assessing Bt protein residues

Received: November 21, 2012
Revised: January 17, 2013
Accepted: January 17, 2013
Published: January 17, 2013

in soil using response surface methodology (RSM) optimization. The RSM was first introduced to optimize chemical reaction conditions and process parameters, and it has been successfully used as an optimizing technique in analytical method development.[24,25] As one of the classic RSM experimental design methods, Box-Behnken design (BBD) is based on three-level partial factorial designs and its experimental points are located on a hypersphere equidistant from the central point.[26] In the current study, a three-level-three-factor BBD was applied for investigating the interactions between the critical extraction variables including extraction temperature, rotation speed, and extraction time to achieve the highest extraction efficiency for Cry1Ac protein from soil.

MATERIALS AND METHODS

Chemicals and Reagents. The standard of Cry1Ac protein was purchased from EnviroLogix Inc. (Portland, ME). Two cotton varieties including a nontransgenic cotton isoline Simian 3 and a transgenic cotton GK 12 cultivar, were used in the current study. The transgenic cotton GK 12 cultivar, which contained the synthetic version of the insecticidal Cry1Ac toxin gene from Bt subsp. *Kurstaki*, was generated by importing the synthetic GFMCry1A insecticidal genes into the nontransgenic cotton isoline Simian 3 through a pollen tube pathway method. Both GK 12 and Simian 3 cotton seeds were supplied by the Cotton Research Institute of Chinese Academy of Agricultural Sciences (Anyang, China). The two cotton varieties have been commercially planted in northern China and were collected from the fields where no Bt insecticides had ever been applied during the entire cotton-growing seasons.

Concentrations of the Cry1Ac protein in the extracts were quantified by ELISA using a commercially available AP 003 Quantiplate kit (EnviroLogix), which was commonly used for Cry1Ab/Cry1Ac proteins analysis.

Chemical grade Tween-20 and concentrated sulfuric acid were purchased from Guangzhou Chemical Company (Guangzhou, China), while analytical grade sodium dodecyl sulfate (SDS) was obtained from Tianjin Chemical Reagent Company (Tianjin, China). The Milli-Q water (Millipore, Bedford, MA) was used to prepare the buffers.

Soil Collection and Spiking. The optimization of the extraction method was conducted using a reference soil collected from a garden in Nanjing Institute of Environmental Sciences, Jiangsu, China, where neither genetically modified plants have been planted nor Bt insecticides have been applied. The surface soil with a depth of 1−5 cm was sampled using a stainless steel spade. The soil was air-dried at room temperature, grounded and passed through a 300 μm sieve, and stored at 4 °C in the darkness before use. The soil pH was 7.47 ± 0.01, and the contents of organic matter, total nitrogen and total phosphorus of the soil were 19.9 ± 0.25, 1.0 ± 0.10 and 0.94 ± 0.02 g/kg, respectively.

The soil was spiked with appropriate amounts of the crude Bt protein solutions which were extracted from the GK 12 transgenic cotton seeds using a PBST solution following the method recommended by EnviroLogix company. The PBST solution was a phosphate-buffered saline (PBS: 1.9 mmol/L NaH_2PO_4, 8.1 mmol/L Na_2HPO_4, 150 mmol/L NaCl, pH 7.4) containing 0.55% of Tween-20 and the solution could be stored for two months at 4 °C. The GK 12 seeds were delinted with concentrated sulfuric acid and then grounded to powder with the addition of liquid nitrogen. For each seed, 2 mL PBST solution was added to the seed powder, slowly grinded into pulp, filtered to remove the testa and organization, and then centrifuged at 12 000 rpm for 3 min at −4 °C. Finally, the supernatant was decanted and used as the crude Bt protein. The soluble Cry1Ac protein concentration in the crude Bt protein solution was measured using an ELISA method described later. After spiking with appropriate amounts of the freshly prepared Bt crude protein solution, the soil was thoroughly mixed on a micro-oscillator (IKA-Werke GmbH & Co. KG, Staufen, Germany). Milli-Q water was added to the soil samples to ensure the moisture content was 20% for all of the samples.

Furthermore, two additional soil samples were collected from Minghang New Village in Nanyang, Yancheng, Jiangsu, China. The two soils were collected from the cotton fields where Bt cottons have been cultivated for 8 and 12 years, respectively. The soil samples were collected right after the cotton harvest in the autumn and placed into sterile bags, sealed and stored at 4 °C in the darkness.

Quantification of Bt Proteins by ELISA. Concentrations of Cry1Ac protein in the extracts were determined by ELISA using a commercial Cry1Ac protein Quantiplate kit AP 003. The kit contained a 96-well solid microplate which was precoated with a CrylAc antibody. Absorbance was measured at the wavelength of 450 nm using a Varioskan Flash Spectral Scan Multimode Plate Reader (Thermo Fisher Scientific, Waltham, MA). External standard calibration was used to quantify the concentrations of CrylAc protein in the extracts and the calibration curves were established using Cry1Ac standard at concentrations ranged from 0.05 to 5 ng/mL.

Optimization of Extraction Method. Three-level-three-factor BBD was used to optimize extraction conditions for analyzing Cry1Ac protein in soil. Since extraction temperature (X_1), rotation speed (X_2), and extraction time (X_3) would significantly influence extraction efficiency, they were chosen as the critical variables to be optimized to achieve the highest extraction yield (Y).

The solution used to extract Bt proteins from soil samples was similar as the PBST solution which was mentioned earlier and used to extract Bt proteins from cotton seeds, with the modification of adding 0.1 mg/mL SDS to the PBST solution (the PBST/SDS solution). Before extraction, approximate 0.4 g soil (dry weight (dw)) was placed into a centrifuge vial and vortexed with 1.2 mL of PBST/SDS solution for 30 s. Then the extraction was conducted at varying temperature (20 to 30 °C), rotation speed (500 to 1100 rpm) and extraction time (1 to 3 h) to find the optimal extraction conditions. Extraction yield (Y) was calculated according to the method outlined by Shan et al.,[21] and the ranges of the independent variables (X_1, X_2, and X_3) were selected according to the preliminary single-factor-test. A total of 15 treatments were carried out according to the BBD experimental design, and low, middle, and high levels of the coded values were designated for the variables as 1, 0, and −1, respectively. The coded and actual levels of the independent variables in the BBD experimental design matrix were listed in Table 1.

The BBD optimization process generally involves performing the statistically designed experiments, estimating the coefficients in the mathematical model, predicting the responses, and verifying the adequacy of the model.[27] In the current study, the BBD experimental design, statistical analysis of variance (ANOVA), regression model analysis, as well as drawing the three-dimensional response surface graphs and the two-dimensional contour plots were all performed using the statistical analysis software STATISTICA 8.0 (StatSoft Inc., Tulsa, OK).

In the BBD experimental design, the independent variable x_i was coded as X_i, which was a dimensionless term as shown in eq 1.

$$X_i = (x_i - x_0)/\Delta x_i \quad (1)$$

where, X_i is the coded value of an independent variable, x_i is the actual value of an independent variable, x_0 is the actual value of an independent variable in the center point, and Δx_i is value of the step change.

The response variable (Y) was fitted to a second-order polynomial model as follows:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j \quad (2)$$

where, Y is the predicted response (extraction yield), β_0 is the intercept, β_i is the slope (i.e., the linear effect) of the independent variable x_i, β_{ii} is the quadratic effect of the independent variable x_i, while β_{ij} is the linear by linear interaction effect between the two independent variable x_i and x_j. Additionally, k represents the number of the variables.

All of the extraction tests were conducted in triplicate and the coefficients of the mathematical model were determined. Next, the

Table 1. Coded and Actual Values of the Independent Variables in the Box-Behnken Design Matrix with the Experimental and Predicted Values of Extraction Yield of Cry1Ac Protein from Soil[a,b]

	code variables [a]			actual values			extraction yield (Y) %	
test	X_1	X_2	X_3	x_1	x_2	x_3	experimental	predicted
1	−1	−1	0	20	500	2	24.6 ± 1.9	23.7
2	1	−1	0	30	500	2	48.7 ± 1.6	47.4
3	−1	1	0	20	1100	2	42.1 ± 0.8	43.5
4	1	1	0	30	1100	2	30.6 ± 1.9	31.6
5	−1	0	−1	20	800	1	27.2 ± 0.3	24.9
6	1	0	−1	30	800	1	45.6 ± 2.6	43.7
7	−1	0	1	20	800	3	32.3 ± 2.1	34.2
8	1	0	1	30	800	3	24.9 ± 2.0	27.2
9	0	−1	−1	25	500	1	18.3 ± 1.5	21.6
10	0	1	−1	25	1100	1	33.1 ± 1.7	34.1
11	0	−1	1	25	500	3	29.5 ± 3.6	28.5
12	0	1	1	25	1100	3	23.3 ± 0.5	20.0
13	0	0	0	25	800	2	44.1 ± 2.9	44.8
14	0	0	0	25	800	2	47.3 ± 1.2	44.8
15	0	0	0	25	800	2	42.9 ± 4.2	44.8

[a]The experiment extraction yields were shown as means ± standard deviation of three replicates. [b]The coded variables X_1, X_2, and X_3 were calculated from their respective real values x_1, x_2, and x_3 using the equations $X_1 = (x_1 - 25)/5$, $X_2 = (x_2 - 800)/300$ and $X_3 = (x_3 - 2)/1$, and X_1, X_2, and X_3 corresponded to the independent variables including extraction temperature (°C), rotation speed (rpm), and extraction time (h), respectively.

optimum conditions of extraction temperature (X_1), rotation speed (X_2), and extraction time (X_3) were selected through the BBD analysis. Ultimately, the accurate optimal values of the variables for extracting Cry1Ac protein from soil were obtained using numerical computation software MATLAB 7.0 (Mathworks Inc., Natick, MA, USA).

Validation and Application of Extraction Method. The final step of the BBD optimization process was the verification of the adequacy of the model.[27] In order to verify the accuracy of the model, three additional experiments were conducted under the optimized conditions, and the experimental extraction yield was compared to the predicted value from the model.

Moreover, the method detection limit (MDL) was determined to assess the sensitivity of the method. The MDL was defined as the minimum level of a chemical that can be identified, quantified, and reported with 99% confidence that the concentration of the analyte was greater than zero.[28] The MDL was derived from the standard deviations of seven measurements of Cry1Ac protein from soil which was spiked with 2 ng/g dw of the protein. The calculation was as the following equation:

$$\mathrm{MDL} = t_{(n-1,1-\alpha=0.99)} \times \mathrm{SD} \quad (3)$$

where, SD is the standard deviation of the measurements, $t_{(n\text{-}1,1\text{-}\alpha=0.99)}$ is the Student's t-distribution value taken at a confidence level of 0.99 and a degree of freedom of $n-1$, and n is the number of the measurements. A t value of 3.14 is used for the testing with seven replicates.

At last, two soil samples from the cotton fields where Bt cottons have been cultivated for multiple years were analyzed using the newly established extraction method.

RESULTS AND DISCUSSION

Extraction Method Development. Palm et al.[20] suggested that both ionic and hydrophobic interactions were important for extracting Bt proteins from soil and a saline solution with Tween-20 was an effective extraction solution. In the current study, a similar extraction solution was used but with 0.1 mg/mL SDS being added. On one side, as an anionic surfactant, SDS might interact with protein molecules through electrostatic interaction to form water-soluble complexes and promote extraction efficacy of the proteins. On the other side, SDS might also interact with the nonpolar groups in the protein molecules and change the conformation of the protein molecules, resulting in protein denaturation.[29] As a consequence, low concentrations of SDS benefited the extraction, but high concentrations of SDS reduced the extraction yield of Cry1A protein. Hence, a small amount of SDS (0.1 mg/mL) was added to the PBST solution and extraction yield was improved approximate 5−8% after the addition of SDS.

In addition to the extraction solution, extraction temperature, rotation speed, and extraction time also played key roles on extraction yield, thus the BBD optimization process was applied to find the optimal extraction conditions.

Model Fitting and Statistical Analysis. A second-order polynomial regression equation with the inclusion of interaction terms was used to fit the experimental data obtained from the BBD experimental design using multiple nonlinear regression analysis (eq 4).

$$Y = 44.7667 + 2.9500X_1 - 0.9083X_1^2 + 1.0860X_2 - 7.3583X_2^2 - 1.7750X_3 - 11.3583X_3^2 - 8.9000X_1X_2 - 6.4500X_1X_3 - 5.2500X_2X_3 \quad (4)$$

As shown in Table 1, the experimental Y values fitted well with the predicted Y values which were calculated using the regression model (eq 4). It demonstrated the feasibility to apply BBD to setting up the experimental optimization design for extracting Cry1Ac protein from soil and establishing the regression equation model.

The p values were used as a tool to check the significance of the interactions between the variables. A p value less than 0.05 indicated that the coefficient was statistically significant. It can be seen from Table 2 that two quadratic term coefficients (X_2^2 and X_3^2) and three interaction coefficients (X_1X_2, X_1X_3, and X_2X_3) were significant and the other term coefficients were not significant. As shown in Table 3, ANOVA analysis for the

Table 2. Regression Coefficients and Their Significances in the Second-Order Polynomial Regression Equation for Extraction Yield (Y) of Cry1Ac Protein from Soil Using a Box-Behnken Experimental Design[a]

model term	estimate	degree of freedom (DF)	standard error (SE)	t value	p value
intercept	44.767	1	1.956	22.890	<0.0001
X_1	2.950	1	1.198	2.463	0.057
X_1^2	−0.908	1	1.763	−0.515	0.628
X_2	1.086	1	1.198	0.835	0.441
X_2^2	−7.358	1	1.763	−4.174	0.009
X_3	−1.775	1	1.198	−1.482	0.198
X_3^2	−11.358	1	1.763	−6.443	0.001
X_1X_2	−8.900	1	1.694	−5.255	0.003
X_1X_3	−6.450	1	1.694	−3.808	0.013
X_2X_3	−5.250	1	1.694	−3.100	0.027

[a]X_1, X_2, and X_3 corresponded to the independent variables of extraction temperature, rotation speed, and extraction time, respectively. Statistical significance was set at 95% of confidence level ($p < 0.05$).

Table 3. Variance Analysis of the Regression Equation[a]

source	sum of square (SS)	degree of freedom (DF)	mean square (MS)	F value	p value
model	1329.55	9	147.73	12.87	0.00082
residual	57.37	5	11.47		
lack of fit	47.02	3	15.68	3.03	0.26
pure error	10.35	2	5.17		
total	1386.92	14			

[a] R^2 = 0.96 and adjusted R^2 = 0.88.

model suggested that the predicted model reasonably represented the experimental values. A model F value of 12.87 implied that the model was significant and a model p value of <0.001 further confirmed that the model was suitable for use in the current experiment. Moreover, the p value for lack of fit of this model was 0.26, which indicated that lack of fit was insignificant relative to the pure errors. This also supported the utility of the model from the other side.

In addition, goodness of the model was double checked by the coefficient of determination (R^2) and the adjust coefficient of determination (Adj R^2). The R^2 and Adj R^2 values of the predicted model were 0.96 and 0.88, respectively, indicating that 96% of the variability in the response could be explained by the predicted model. Overall, statistical analysis reflected that the experimental values fitted well with the predicted ones and the accuracy and general availability of the polynomial model were adequate for further optimization.

Selection of Optimal Conditions with Response Surface Graphs. Both three-dimensional response surface and two-dimensional contour graphs were the graphical representations of regression functions. While the former described the sensitivity of response value toward the change of variable, the latter illustrated the interactions between the corresponding variables are significant or not. Figures 1−3 showed the interactions between extraction temperature and rotation speed, extraction temperature, and extraction time, as well as rotation speed and extraction time, respectively. All of the graphs were plotted by fixing one variable at a constant level, which was the coded zero level, while the other two variables varied within the experimental ranges. The difference in the contour plot shapes indicated different interactions between the variables[30] and the optimal values of the variables were selected when the response reached the acme.

The response surface and contour plots, Figure 1a,b, respectively, showed the effects of extraction temperature and rotation speed on extraction yield when extraction time was fixed at 2 h. Figure 1 indicated that extraction temperature displayed a linear effect on the response. At low rotation speed, extraction yield increased when extraction temperature increased; however, the opposite trend was observed when rotation speed exceeded a certain level, that is, extraction yield decreased with the increase in extraction temperature. On the contrary, rotation speed had a quadratic effect on extraction yield.

The relationship among extraction yield, extraction temperature and extraction time at a fixed rotation speed of 800 rpm was plotted Figure 2. A linear effect of extraction temperature and a quadratic effect of extraction time on extraction yield were observed. The influence of extraction temperature on extraction yield was similar as that shown in Figure 1a. The symmetrical shape in Figure 2a indicated that extraction yield

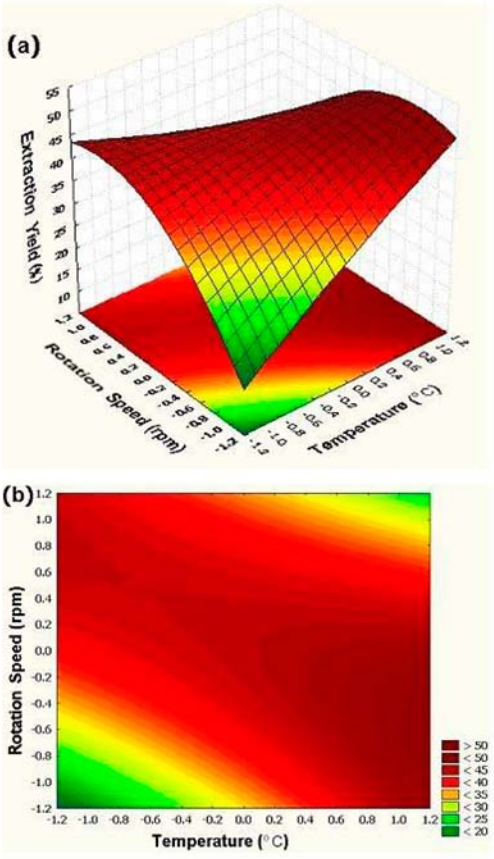


Figure 1. Response surface (a) and contour (b) graphs of the combined effects of extraction temperature and rotation speed on extraction yield when extraction time was held at zero level.

initially increased with increasing extraction time at the beginning of extraction, but slightly decreased thereafter.

Similarly, Figure 3a,b were the response surface and contour plots, respectively, depicting the effects of rotation speed and extraction time on extraction yield with a fixed temperature of 25 °C. From Figure 3a, we can conclude that both rotation speed and extraction time displayed quadratic impacts on the response and this was consistent with the conclusions drawn from Figures 1 and 2. Extraction yield initially increased and then declined with continuing increasing rotation speed from 800 to 1100 rpm. Consequently, there was an optimal value for rotation speed which corresponded to the highest extraction yield. Similarly, extraction yield increased rapidly when extraction time extended from 1 to 2 h, reaching a plateau region with a maximum value, and then slightly decreased when extraction continued from 2 to 3 h. The optimum values of the variables were located within the experimental ranges, implying the effectiveness of using the response surface analysis method to identify the optimal conditions.[24] The slope of the response surface in Figure 3a was relatively flat, indicating that the interaction of rotation speed and extraction time was not significant. Contour shape also reflected the interaction effects between two independent variables. Figures 1b and 2b demonstrated that interaction of extraction temperature with the other two independent variables (rotation speed and extraction time) was significant, whereas the interaction between the latter two variables was relatively insignificant (Figure 3b).

dx.doi.org/10.1021/jf304970g | J. Agric. Food Chem. 2013, 61, 1464−1470

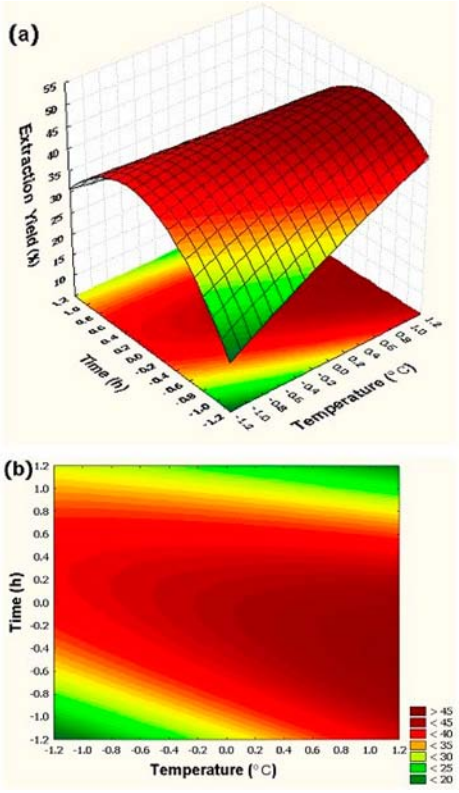


Figure 2. Response surface (a) and contour (b) graphs of the combined effects of extraction temperature and time on extraction yield when rotation speed was held at zero level.

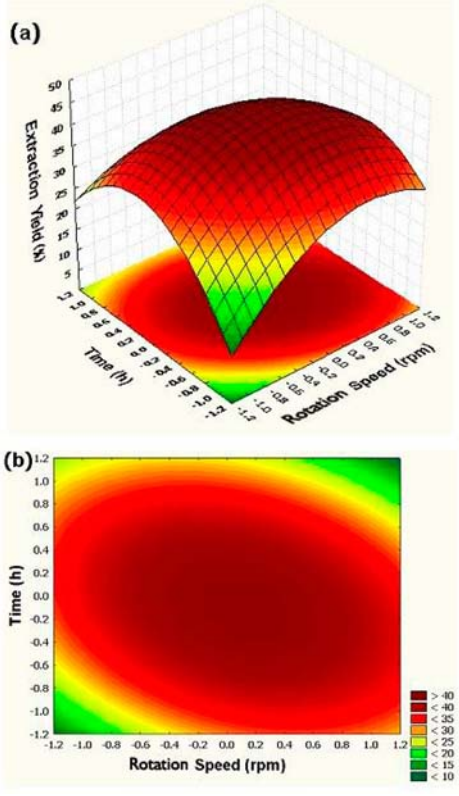


Figure 3. Response surface (a) and contour (b) graphs of the combined effects of rotation speed and extraction time on extraction yield when extraction temperature was held at zero level.

Ultimately, the optimal values of the variables for extracting Cry1Ac protein from soil were computed and the suitability of the model equations for predicting optimum response values was tested. The optimal condition to extract Cry1Ac protein from soil was using PBST/SDS solution, which was a phosphate-buffered saline containing 0.55% Tween-20 and 0.1 mg/mL SDS, as the extraction solution, and the extraction was conducted at temperature of 21 °C, rotation speed of 630 rpm for 2 h. Under the optimal conditions, the predicted extraction yield from the model was 47.7%.

Method Validation and Application. To validate the accuracy of the model to predict extraction yield, Cry1Ac protein was extracted from the spiked soil in triplicate under the optimal conditions. Extraction yield calculated from the experimental measurements was 46.5 ± 3.4%, which was in good agreement with the predictive value of 47.7%. The good fitting of the experimental and predicted values further supported that the second-order polynomial model used in the current study was adequate for describing the extraction process of Cry1Ac protein from soil.

The extraction yield of 47% in the current study was within the range of those previously reported.[18,20−23,31,32] The extraction procedure developed by Palm et al.[20] was used to analyze Bt proteins from the soil samples collected from Bt transgenic rice fields in China, and extraction yields of 4.6−35% were reported.[32] Recently, extraction with an artificial gut fluid which was formulated to mimic a marine worm gut fluid has been proposed and showed a good efficiency of 88% for extracting Bt proteins from soil, however, the requirement of adding the artificial worm protein ingredients to the extraction solution increased the costs of method.[31] Although extraction efficiency was good, detection limit of the gut fluid extraction method was not satisfactory with a MDL of 4.5 ng/g dw.[31]

The characteristics of the soil samples played a key role in extraction yield of Bt proteins. It has been shown that extraction yield differed significantly when soils had different mineralogy and clay contents.[18,22,23] The greater amounts of the clay contents, the tighter Bt proteins being sorbed to the soil. As a result, the proteins were more difficult to extract from the soil.[33] Gruber et al.[18] has reported a strong correlation between the recoveries of Bt proteins and clay contents in soil. In their study, extraction recovery was only 49% when the clay content exceeded 25%, but it jumped to 85% when the clay content dropped to less than 6%.[18] In the current study, the soil contained approximate 2% of organic matter and the soil was sieved through the 300 μm sieve before use, thus the clay content in the soil may be high. With relatively high clay content, the binding between Cry1Ac protein and the soil was strong, and this may be one of the reasons for only half of the proteins being extracted from the soil. Therefore, future study is required to better understand the interactions between Cry1Ac protein and the clay in order to conducting more effective extraction. Previous studies implied that extractability was correlated to the intracrystalline swelling of the clay.[34,35] The selection of appropriate extraction solution and prewetting the soil samples would benefit the swelling process and facilitate the penetration of extraction solution into the interstices of the clay, consequently improve the extraction recovery.[35]

Detection limit of the established method was evaluated with the MDL, and the MDL calculated from the standard derivation of seven replicate measurements was 0.8 ng/g dw. The MDL was comparable to that reported by Palm et al. (0.5 ng/g dw)[20] while lower than those by Shan et al. (4.5 ng/g

dw),[31] Head et al. (3.68 ng/g dw using ELISA and 8 ng/g dw using bioassay),[15] and Gruber et al. (2 ng/g dw).[18] Additionally, precision of the method was indicated by the relative standard deviation (RSD) of the experimental replicates. With a RSD of 7.3%, the current method was precise.

At last, three field-collected soils were analyzed under the optimal conditions derived from the BBD model. The three soil samples included one soil from the field where no transgenic crops have ever been planted and the other two soils from cotton fields where Bt transgenic cottons have been cultivated for 8 and 12 years before collecting the samples. Concentrations of Cry1Ac protein extracted from the soils were shown in Table 4. No Cry1Ac protein was detected in the soil without

Table 4. Concentrations of Cry1Ac Protein in Field-Collected Soils with or without History of Bt Cottons Cultivation[a,b,c,d,e]

soil	concentration (ng/g dry weight)	relative standard deviation (%)
not-Bt	ND b	/
Bt-8	0.90 ± 0.04 a	4.4
Bt-12	0.94 ± 0.08 a	8.5

[a]The experiment was conducted in triplicate and concentration was shown as the mean ± standard derivation. The different letter indicated significant difference ($p < 0.05$). [b]Not-Bt: The soil from the field where no genetically modified crops have ever been planted. [c]Bt-8: The soil from the field where transgenic crops have been planted for eight years. [d]Bt-12: The soil from the field where transgenic crops have been planted for 12 years. [e]ND: Not detectable with a method detection limit of 0.8 ng/g dry weight.

Bt crops even being planted. On the contrary, 0.90 ± 0.04 and 0.94 ± 0.08 ng/g dw of the protein was detected in the soils where Bt cottons were planted for 8 and 12 years, respectively. Though trace Cry1Ac protein was detected in the soils, the concentrations were just above the MDL and below the method quantification limit which was considered as 3-fold of MDL. Moreover, there was no significant difference in the levels of Cry1Ac protein residues in the two soils although they had different lengths of transgenic crops planting history.

Trace amounts of Bt proteins have also been reported in soils after cultivation of Bt maize and the measured concentrations ranged from <0.1 to 3 ng/g dw.[36,37] Conversely, Head et al.[15] and Gruber et al.[18] reported no detection of Bt protein residues in soils after multiple years of planting Bt crops. The discrepancy in these studies[15,18,36,37] as well as the current study may be the result of the difference in the MDLs of different methods and the extremely low concentrations of Bt protein residues in the field.

In conclusion, a three-level-three-factor BBD was used to optimize the conditions to extract Cry1Ac protein from soil. Response surface methodology was a useful tool in optimizing extraction conditions. It was using PBST/SDS as extraction solution and conducting extraction at 21 °C for 2 h with a rotation speed of 630 rpm. The developed method was precise and sensitive with the MDL of 0.8 ng/g dw, but only half of the soil-associated protein was extracted. High clay content in the soil may be one of the reasons for the relatively low recovery. Although trace levels of Cry1Ac protein were detected in soils where Bt crops have been cultivated for multiple years, no significant difference was observed for the soils with different lengths of planting history.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Financial support from the Chinese Academy of Sciences ("Interdisciplinary Collaboration Team Program" and GIGCAS-135-project Y23402100x) and the National Special Transgenic Project (2011ZX08011-002 and 2011ZX08012-005) is thankfully acknowledged. GIGCAS contribution no. 1614.

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